Collection and Analysis of Salivary Proteins from the Biting Midge *Culicoides nubeculosus* (Diptera: Ceratopogonidae)

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ABSTRACT Salivary proteins of hematophagous *Culicoides* spp. are thought to play an important role in pathogen transmission and skin hypersensitivity. Analysis of these proteins, however, has been problematic due to the difficulty in obtaining adequate amounts of secreted *Culicoides* saliva. In the current study, a collection method for midge saliva was developed. Over a 3-d period, 3- to 5-d-old male and female *Culicoides nubeculosus* Meigen (Diptera: Ceratopogonidae) were repeatedly placed onto the collection system and allowed to deposit saliva into a filter. Salivary products were eluted from the filters and evaluated by gel electrophoresis and mass spectrometry as well as by intradermal testing and determination of clotting time. Gel electrophoresis revealed ≈55 protein spots displaying relative molecular masses from 5 to 67 kDa and isoelectric points ranging from 4.5 to 9.8. The majority of molecular species analyzed by mass spectrometry showed high convergence with salivary proteins recently obtained from a cDNA library of *Culicoides sonorensis* Wirth & Jones, including proteins involved in sugarmeal digestion, defense, and coagulation inhibition as well as members of the D7 family and unclassified salivary proteins. In addition, the proteome analysis revealed a number of peptides that were related to proteins from insect species other than *Culicoides*. Intradermal injection of the saliva in human skin produced edema, vasodilatation, and pruritus. The anticoagulant activity of the saliva was demonstrated by significantly prolonged clotting times for human platelets. The potential role of the identified salivary proteins in the transmission of pathogens and the induction of allergies is discussed.

KEY WORDS *Culicoides nubeculosus*, saliva collection, proteomics, vasodilatation, coagulation inhibition

Culicoides biting midges are competent vectors of a wide range of economically important pathogens that affect both domestic and wild animals (Mellor et al. 2000). Midges can transmit bluetongue virus (Chandler et al. 1985), epizootic hemorrhagic disease virus (Paweska et al. 2005), vesicular stomatitis virus (Drolet et al. 2005, Perez de Leon and Tabachnick 2006), and African horse sickness virus (Mellor and Hamblin 2004). In addition to acting as a vector, *Culicoides* spp. are the primary cause of an extremely pruritic allergic dermatitis known colloquially as "sweet itch," "summer eczema," "insect bite hypersensitivity," "Queensland itch," and "Kasen" in atopic horses worldwide (Riek 1953, Anderson et al. 1993, Kurotaki et al. 1994, Marti et al. 1999, Mckelvie et al. 1999).

It has been suggested that the saliva of hematophagous arthropods plays an important role in mediating pathogen transmission (Ribeiro and Francischetti 2003). This role is illustrated by the mechanism of saliva-activated transmission, first reported for tick-borne encephalitis (Jones et al. 1990). In addition, pathogens such as *Leishmania* spp., vesicular stomatitis virus, dengue fever virus, and Sindbis virus have shown enhanced or decreased infections of mammalian hosts or cell lines when coadministered with the saliva of the pathogen's specific vector (Titus and Ribeiro 1988; Theodos et al. 1991; Limesand et al. 2000, 2003; Ader et al. 2004; Schneider et al. 2004). Vector saliva also may be influential in arbovirus transmission from one vector to another by local cofeeding on infected nonviraemic mammalian hosts (Labuda et al. 1993, 1997; Mead et al. 2000; Higgs et al. 2005). Moreover, saliva of hematophagous insects is known to be an important source of allergens (Galindo et al. 1998, McDermott et al. 2000, Paddock et al. 2001). Salivary

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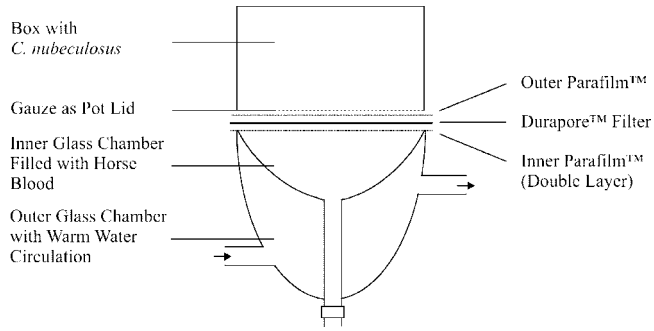

Fig. 1. Saliva of *C. nubeculosus* was obtained using a modified artificial membrane feeding system. Midges deposited saliva proteins in the Durapore filters attracted by 37°C horse blood in the inner glass chamber. Contamination with nonsaliva products was avoided by covering the filters with Parafilm.

proteins isolated from the mosquito *Aedes aegypti* (L.) induced an allergic dermatitis in atopic humans (Peng and Simons 2004). Bites of the cat flea, *Ctenocephalides felis* Bouche, cause a similar disease in cats and dogs (Lee et al. 1999, Bond et al. 2006). The saliva of assassin bugs (Triatominae) contains several allergens responsible for severe allergic reactions such as urticaria, dyspnea, and life-threatening anaphylaxis in humans (Moffitt et al. 2003).

Despite their relevance for pathogen transmission and hypersensitivity, individual salivary proteins of *Culicoides* spp. have not yet been biochemically identified. Fractionation of *Culicoides sonorensis* Wirth & Jones salivary glands has revealed that midge saliva possesses functional components commonly found in hematophagous insects such as those preventing blood coagulation and promoting vasodilatation (Perez de Leon and Tabachnick 1996; Perez de Leon et al. 1997, 1998). Recently, a broad range of genes encoding salivary proteins were characterized from a salivary gland cDNA library of *C. sonorensis* and several proteins involved in roles such as hemostasis, pheromone binding, and digestion were identified (Campbell et al. 2005). However, because the levels of mRNA and expressed gene products often do not correspond, it is necessary to analyze the proteome itself (Anderson and Seilhamer 1997).

Thus far, the lack of sufficient quantities of purified midge saliva has restricted research on *Culicoides* salivary proteins. Midge saliva has been collected to demonstrate the secretion of bluetongue virus by individual *C. sonorensis*, but the method is not suitable for the collection of larger amounts (Boorman 1987, Fu 1995, Fu et al. 1999). Here, we report the application of a novel, quick, and efficient collection method modified from an established artificial membrane feeding system used to experimentally infect *Culicoides* spp. with pathogens (Mellor 1971, Venter et al. 1991) and a saliva collection apparatus developed for the cat flea (Frank et al. 1996). We describe the first identification of several individual midge salivary proteins obtained with the novel collection method. In addition, the collected saliva has demonstrated the preserved functional properties of induction of skin reactivity and inhibition of platelet aggregation. Development of this saliva collection method and initial analysis and identification of midge salivary proteins will help further the field of salivary proteomics as well as investigations on virus transmission and hypersensitivity for *Culicoides* spp.

Materials and Methods

Saliva Collection and Elution of Proteins. A preexisting membrane feeding system reported by Mellor (1971) was modified for saliva collection from 2- to 5-d-old, unfed *Culicoides nubeculosus* Meigen (Diptera: Ceratopogonidae) reared according to the method of Boorman (1974). The collection system consisted of an inner and an outer glass chamber (Fig. 1). The inner chamber was filled with horse blood and sealed with a double layer of Parafilm membrane (American National Can, Greenwich, CT). The outer chamber was connected to a heated water circulation system to adjust the blood temperature to 37°C. A hydrophilic Durapore filter (Millipore, Eschborn, Germany), similar to that described for the collection of salivary proteins from the cat flea (Frank et al. 1996), was used in the collection system. The filter was soaked in sucrose solution (10% wt:vol) to encourage midge feeding and placed on top of the Parafilm membrane. Finally, a third layer of Parafilm was placed above the filter to prevent contamination with nonsalivary products of the midges.

More than 5,000 *C. nubeculosus* (male and female) in 20 pill boxes (Watkins and Doncaster, Kent, United Kingdom) covered with fine mesh gauze were allowed to probe through the membrane and to deposit saliva on the filter. Each box contained 250–300 insects and was placed on the saliva collection system for 20–30 min, two to three times a day (4–6-h intervals) over a period of 3 d. Filters were changed when dry and stored at 4°C in phosphate-buffered saline (PBS) until further processing. For the elution of salivary products, filters were shaken overnight at 4°C. The eluate was concentrated to a volume of 1 ml by using ultrafiltration tubes (cut-off 3 kDa, Vivaspin 20, Vivascience, Hannover, Germany). The concentrate was desalted by subsequent addition of 30 ml of MilliQ water (Millipore) and centrifuged back to a volume of

1 ml. The bicinchoninic acid protein assay (Perbio Science, Bonn, Germany) was used to determine the protein concentration. The concentrate was stored at 4°C. Before gel electrophoresis the required volume was dried in a vacuum centrifuge.

One-Dimensional (1D) Gel Electrophoresis. Vacuum-dried salivary proteins (20 μg per lane) were dissolved in 1× sodium dodecyl sulfate (SDS) sample buffer (100 mM dithiothreitol [DTT], 62.5 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 0.2% Bio-Lyte ampholytes [Bio-Rad, Munich, Germany], and 0.001% bromphenol blue). SDS-polyacrylamide gel electrophoresis (PAGE) (Laemmli 1970) was done using 1-mm-thick 15% polyacrylamide gels in a MINI PROTEAN II Cell (Bio-Rad). Gels were run with SDS running buffer (192 mM glycine, 25 mM Tris, and 0.1% SDS) at 200 V. The Mark12 wide range ladder (Invitrogen, Karlsruhe, Germany) was used to determine molecular masses of salivary proteins.

Two-Dimensional (2D) Gel Electrophoresis. To separate proteins by their isoelectric points (pIs), vacuum-dried salivary proteins (50 μg per strip) were dissolved in 125 μl of rehydration buffer containing 8 M urea, 2% 3-[(3-cholamido-propyl) dimethylammonio]-1–1propanesulfonate (Omnilab, Hannover, Germany), 40 mM DTT, 0.2% Bio-Lyte 3–10 (Bio-Rad), and 0.001% bromphenol blue. Samples were applied to immobilized pH gradient (IPG) 7-cm strips (pH 3–10 and 3–6, Bio-Rad) by passive rehydration for 12 h. Isoelectric focusing was performed at a voltage of 4,000 V until 10,000 Vh were reached. Before running the SDS-PAGE for the second dimension, the IPG strips were soaked for 15 min in DTT equilibration buffer (6 M urea, 0.375 M Tris-HCl, 130 mM DTT, 20% [wt:vol] glycerol, and 2% [wt:vol] SDS, pH 8.8) followed by 15 min in iodoacetamide equilibration buffer (6 M urea, 0.375 M Tris-HCl, 135 mM iodoacetamide, 20% [wt:vol] glycerol, and 2% [wt:vol] SDS, pH 8.8). Second-dimension electrophoresis was done as described for 1D gels.

In-Gel Digestion and Peptide Extraction. After 1D and 2D electrophoresis, proteins were visualized using the Gelcode Snap Silverstain kit (Perbio Science). When in-gel protein digestion was to be performed, gels were stained with Simply-Blue-Stain (Invitrogen, Carlsbad, CA). Evaluation of molecular masses and isoelectric points was done arithmetically. Protein spots stained with Simply-Blue-Stain were excised and transferred to 1-ml tubes that had been previously rinsed with MilliQ water and acetonitrile to remove any contaminants. Gel pieces were washed two times for 15 min with 50 mM ammonium bicarbonate, pH 8.0, and two times for 15 min with 60% acetonitrile/0.5% formic acid until they were completely dehydrated. After drying under vacuum, protein spots were reduced with 10 mM DTT for 20 min at 56°C and alkylated with 100 mM iodoacetamide for 20 min at room temperature in the dark. To remove residual iodoacetamide, gel spots were washed with 50 mM ammonium bicarbonate, pH 8.0, followed by 60% acetonitrile/0.5% formic acid and dried as described above. Protein digestion was performed with trypsin (Promega, Mannheim, Germany) according to the manufacturer's instructions. For peptide extraction, gel pieces were washed with 50 mM ammonium bicarbonate and 60% acetonitrile/0.5% formic acid as described above. The extracts were pooled and concentrated under vacuum to a final volume of 20 μl. ZipTip C18 Sepharose tips (Millipore) were used to desalt and concentrate the peptide samples to a volume of 10 μl.

Electrospray Ionization Quadrupole Time of Flight (QToF)-Tandem Mass Spectrometry and Protein Identification. Peptides eluted from the ZipTip (≈3 μl) were transferred to gold-coated nanospray glass capillaries (Protana, Odense, Denmark). The tip of the capillary was placed orthogonally in front of the entrance hole of a QToF 2 mass spectrometer (Micromass, Manchester, United Kingdom), equipped with a nanospray ion source, and a voltage of ≈1,000 V was applied. For collision-induced dissociation experiments, parent ions were selectively transmitted from the QToF mass analyzer into the collision cell. Argon was used as the collision gas and the kinetic energy was set at −35 eV. Resulting daughter ions were then separated by an orthogonal ToF mass analyzer. The spectra of ions were computer enhanced using Maximum Entropy 3 (Micromass). Amino acid sequences were deduced from carboxy-terminal fragment ion series of the y-type showing amino acid-specific mass increments and were confirmed by complementary aminoterminal fragment ion series of the b-type. The isobaric amino acids isoleucine (I) and leucine (L) were not distinguishable using QToF mass spectrometry; therefore, they were interchangeable in the peptide sequences reported here. Amino acid sequences were used to search the protein database UniProt by using the FASTA program (Pearson and Lipman 1988). The software Align X (Invitrogen) was used for determining alignments, identities of peptide sequences from public databases, and calculations of molecular masses and pI data of database proteins.

Skin Testing of Vasodilatory Activity. Skin testing was performed on humans by using the dorsal aspect of the arms of three volunteers. All subjects were carefully instructed about the experimental procedures and potential risks, and all gave their written consent. The skin region was disinfected with 70% ethanol before application. Saliva samples eluted from the collection filters were adjusted to protein concentrations of 12.5, 25, and 50 μg/ml in PBS. A volume of 50 μl of each dilution was applied intradermally using 27-gauge, 1.25-cm needles and 1-ml syringes. Injection sites were placed ≈2.5 cm apart. PBS was used as negative control. The development of edema and erythema was monitored visually over a 48-h postinoculation (p.i.). The two greatest diameters of the reaction were recorded, and the average was calculated.

Measurement of Human Plasma Clotting Activity. The clotting activity of the salivary proteins extracted from the collection filters was measured by recalcification time with platelet poor plasma (PPP) modified from a method described by Ribeiro et al. (1995). Briefly, a volume of 50 μl of citrated human PPP and

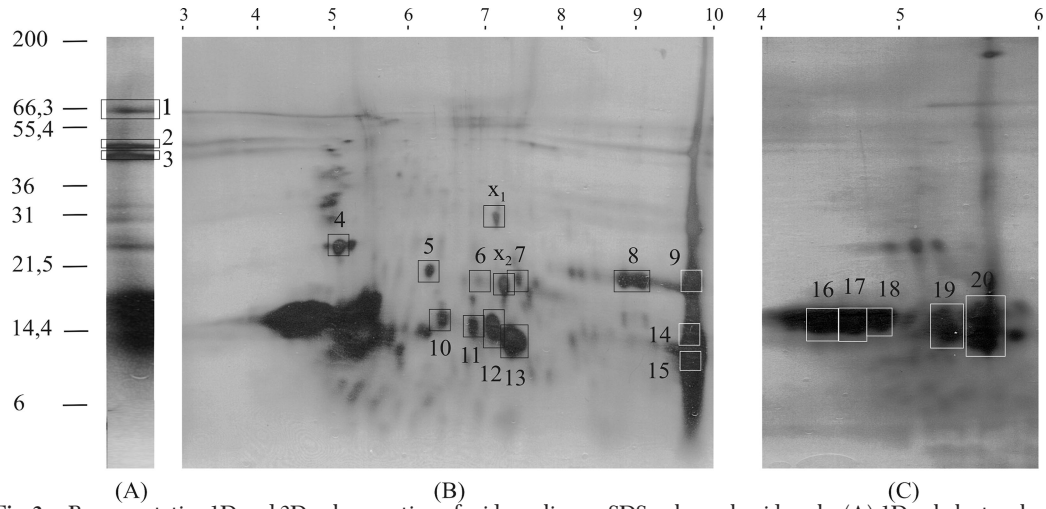

Fig. 2. Representative 1D and 2D gel separation of midge saliva on SDS polyacrylamide gels. (A) 1D gel electrophoresis. (B) 2D gel electrophoresis with IPG-strips in a pH range from 3 to 10. (C) 2D gel electrophoresis with IPG-strips with a pH range from 3 to 6 for a better resolution of acidic, low-molecular-mass proteins (pH 4–6 shown). Gels containing 15% polyacrylamide were used for 1D gel electrophoresis and second dimension of 2D. Molecular masses in kilodaltons are indicated on the left lane, pI data on the top lane. The assigned proteins were used for mass spectrometry. Proteins for these images were visualized by silver staining.

50 μl of 150 mM sodium chloride/10 mM sodium HEPES, pH 7.4, containing salivary proteins at a concentration of 50, 25, and 12.5 μg/ml or no protein (negative control) were mixed in a 96-well flat-bottom plate (Nunc, Wiesbaden, Germany) and maintained at 37°C for 2 min. After the addition of 50 μl of 25 mM calcium chloride to the wells, the plate was placed in a microplate reader (Dynatech, Denkendorf, Germany), heated to 37°C, and mixed. Absorbance readings at 630 nm were recorded every 20 s. A fast, sharp increase in the absorbance after a lag phase indicated clotting. The time taken to reach an absorbance value of 0.1 (onset optical density [O.D.]) was chosen as a measure of clotting time.

Results

Saliva Collection and Elution of Proteins. When midges were placed on the saliva collection system, insects immediately assembled at the gauze–Parafilm interface. Feeding levels were monitored by the extent to which midges crowded together at the interface. The intensity of feeding decreased significantly after 15–20 min and correlated with the engorgement of female midges. Less crowding was observed at the interface when the blood temperature was <37°C. After removal and stretching of the outer Parafilm, numerous tiny holes produced by the bites of midges became visible, whereas the inner membranes remained intact. In total, salivary proteins from >5,000 male and female midges were collected on nine filters over a 3-d period for a total of 1.2 mg of eluted protein.

Protein Analysis on 1D and 2D Gels. 1D gel electrophoresis and staining of eluted proteins revealed three predominant bands at 44, 49, and 66 kDa and three weaker bands at 25, 30, and 33 kDa (Fig. 2A). A large, unresolved area was observed from 10 to 18 kDa. 2D gel electrophoresis using IPG strips with pH ranges of 3–10 displayed ≈55 protein spots with pI values of 4.5–9.8 and molecular masses of 5–67 kDa (Fig. 2B). Acidic low-molecular-weight proteins showed up as a large unresolved area between pI 4 and 6. For better resolution of this region, samples were separated using more focused IPG strips with a pH-range from 3 to 6. Multiple resolved spots were detected between pI 4.5 and 5.7 (Fig. 2C; pH-range 4–6 shown). Based on their reproducibility on multiple 1D and 2D gels, a total of 22 protein spots were selected for identification by MS. The high-molecular-weight proteins (spots 1–3) were analyzed from the 1D gel, because their resolution on 2D gels remained poor.

Protein Identification by Mass Spectrometry. Twenty-two predominant protein spots were selected for internal peptide sequencing by tandem mass spectrometry. Reliable peptide sequences were obtained from 20 protein spots and were used for FASTA analysis to determine the putative identities of these proteins (Table 1). Twelve protein spots showed high convergence with proteins of *C. sonorensis*, recently identified from a salivary gland cDNA library. One of the proteins analyzed from the 1D gel (spot 1) was identified as a maltase (α-glucosidase). Five protein spots (16–20) were related to three different proteins of the D7 family. One protein spot (3), taken from the 1D gel, revealed one peptide that showed high identity to two hypothetical *C. sonorensis* protein fragments involved in defense. One peptide obtained from two individual protein spots (8 and 9) matched with a Kunitz protease inhibitor-like protein involved in coagulation inhibition. Four proteins (2, 3, 6, and 15) were identified as novel or unclassified proteins from *C. sonorensis.* Peptides obtained from three spots (4,

Table 1. Mass spectrometry identification of salivary proteins from *C. nubeculosus*

Spot no.	Peptide sequences[a]	% identity[b]	Protein identification[c], GenBank accession no.	Putative function	Observed[d] M_r/pI	Predicted[e] M_r/pI
1	SFMDSDGDGVGDLK	100	Maltase *Culicoides sonorensis* (*C. s.*), Q66UC5_9DIPT	Sugarmeal digestion (Maltase)	66/n.d.[f]	68/5.0
	VFPQFGDLSSIDELVAQFLER	95				
	NLPPTNWVSAFR	100				
	SSAWEWNEER	100				
	GEYYLHQFLAEQPDLNYR	94				
	VLMVEAYAPLTK	100				
	TVDPQACTTNPNIFHAK	100				
	TPMIWNAQK	78				
	YGTYDSYLANDDVLVIK	100				
	TLIAVLNLGFTEQVVNLNLNER	95				
2	FNQVYFTQEANFGSCNSK	89	Hypothetical protein *C. s.*, Q66U72_9DIPT	Unclassified	49/n.d.	21.7/7.8
3	FNQVYFTQEANFGSCNSK	89	Hypothetical protein *C. s.*, Q66U72_9DIPT	Unclassified	44/n.d.	21.7/7.8
	QLGFTGELYIYGNK	86	Hypothetical protein [Fragment] *C. s.*, Q66TX6_9DIPT	Defense	44/n.d.	25.5/8.1
		86	Hypothetical protein [Fragment] *C. s.*, Q66TX4_9DIPT	Defense	44/n.d.	26.4/8.3
	ALGVELTKFDLSTGK	67	Hypothetical protein [Fragment] *C. s.*, Q66U84_9DIPT	Unclassified	44/n.d.	17.2/9.8
4	FVPTIVYNEQFNQELQDQSLR	76	GA21779-PA [Fragment] *Drosophila* (*D.*) *pseudoobscura*, Q296E2_/DROPS	Unclassified	25/5.1	21.9/5.7
		71	CG9427-PA (GH19763p) *D. melanogaster*, Q9VAH5_DROME	Unclassified	25/5.1	23.4/6.6
		67	ENSANGP00000018265 [Fragment] *Anopheles gambiae*, Q7PR47_ANOGA	Unclassified	25/5.1	17.8/4.7
5	LALVLNSDLFNR		n.i.[g]		21/6.3	
6	GANIQYFTSLK	91	Hypothetical protein *C. s.*, Q66U35_9DIPT	Novel	19/6.9	17.3/6.6
		91	Hypothetical protein *C. s.*, Q66U37_9DIPT	Novel	19/6.9	17.8/6.0
7	VFGVGLYSCPSR	58	Cytochrome P450 monooxygenase *Penicillium paxilli*, Q9C449_PENPX	Cytochrome P450 family	19/7.5	58.8/8.1
		50	Cytochrome P450-like putative monoxygenase *Azoarcus* sp. Q5P8J7_AZOSE	Cytochrome P450 family	19/7.5	18.5/4.7
8	YNVQTNTCFYSLTK	57	Putative secreted salivary protein *C. s.*, Q66U91_9DIPT	Coagulation inhibitor	19/9.0	22.3/9.0
9	YNVQTNTCFYSLTK	57	Putative secreted salivary protein *C. s.*, Q66U91_9DIPT	Coagulation inhibitor	19/9.7	22.3/9.0
10	GSYLSLDGDKETR LLADSTGVPWVLDDDGK VAWSNVMCAFGDWLFVYDR		n.i.		16/6.5	
11	QDAFSVGLYSCLLR LSSPATLNSR LLADSTGVPWVLDDDGK		n.i.		15/6.8	
12	LLADSTGVPWVLDDDGK QDWYYTLENQQYK EANFYVQSR		n.i.		15/7.1	
13	QGDGTELSELPDQSAR	56	Peroxidase precursor *Hordeum vulgare*, O49866 HORVU	Peroxidase	12/7.4	36.5/5.6
		50	Peroxidase precursor *Triticum aestivum*, Q5GMP4_WHEAT	Peroxidase	12/7.4	36.6/5.9

(*continued*)

Table 1. Continued

Spot no.	Peptide sequences[a]	% identity[b]	Protein identification[c], GenBank accession no.	Putative function	Observed[d] M_r/pI	Predicted[e] M_r/pI
14	YPASSASVPWVLDEDG	50	CG7025-PA *D. melanogaster* Q9VL21_DROME	Carboxypeptidase	14/9.7	48.3/4.8
		50	Similar to *D. melanogaster* CG7025 [Fragment] *D. yakuba*, Q6XHU5_DROYA	Carboxypeptidase	14/9.7	21.9/4.7
		50	LP21640p [Fragment] *D. melanogaster*, Q5BI80_DROME	Carboxypeptidase	14/10	48.5/4.8
15	LYQGLLDSYNVLALEQYMK	95	Hypothetical protein *C. s.*, Q66U47_9DIPT, Q66U45_9DIPT, Q66U43_9DIPT	Novel	10/9.7	18.3/8.5
16	ADELYACLLNADGQK	93	D7 related protein *C. s.*, Q66UB7_9DIPT	D7 family	16/4.5	15.3/4.7
	PSGDQYDTDNLLLVR	93				
	DCEEEKDLPA	70				
17	DEAPSGDQYDTDNLLK	81	D7 related protein *C. s.*, Q66UB7_9DIPT	D7 family	15/4.7	15.3/4.7
18	PSGDQYDTDNLLK	100	D7 related protein *C. s.*, Q66UB7_9DIPT	D7 family	16/4.9	15.3/4.7
	VPANPTECYIDCIFQK	88				
19	YYEVENIFQAEWK	96	Putative secreted salivary protein *C. s.*, Q66U41_9DIPT	D7 family	15/5.4	17.8/5.4
20	QCFILCELTNLNILK	93	Putative secreted salivary protein *C. s.*, Q66U39_9DIPT	D7 family	16/5.6	17.8/5.6
	ISECSNLELYQDNCK	60	Chemical sense-related lipophilic ligand-binding homologous protein *Phormia regina* (black blowfly), Q5NTY8_9DIPT		16/5.4	16.4/4.9

[a] Sequence information obtained from peptide fragmentation data. The isobaric amino acids isoleucine (I) and leucine (L) cannot be distinguished using QToF mass spectrometry. Hence, I and L are interchangeable in the peptide sequences reported here.

[b] Calculated identity of obtained peptide sequences to proteins from the database using the AlignX software (Invitrogen).

[c] Proteins were identified by searching the UniProt database with peptide fragmentation data using the FASTA program (European Bioinformatics Institute, Cambridge, United Kingdom).

[d] Observed molecular masses (M_r) in kilodaltons and pIs estimated from protein spots on gel.

[e] Predicted M_r in kilodaltons and pIs from the database.

[f] n.d., not determined.

[g] n.i., not identified.

14, and 20) matched with proteins from insect species other than *C. sonorensis*, including yet unclassified proteins from *Drosophila* spp. and *Anopheles gambiae* Giles, a carboxypeptidase from *Drosophila* spp., and a chemical sense-related lipophilic ligand-binding homologous salivary protein from the black blow fly, *Phormia regina* Meigen. Peptides analyzed from two spots (7 and 13) showed low identities to a fungal and bacterial cytochrome P450 monooxygenase and plant peroxidase precursors. After peptide sequencing, four proteins remained unidentified (spots 5 and 10–12). No reliable sequence data were obtained from two proteins (Fig. 2B, x_1, x_2) and were therefore not included in the 20-spot analysis.

Comparison of observed and theoretical molecular masses and pI data were consistent for the majority of proteins with significant identity to putative *C. sonorensis* salivary proteins. However, the observed masses for two proteins of *C. nubeculosus* (spots 2 and 3) were nearly two times larger than the mass described for the corresponding protein of *C. sonorensis* (Q66U72_9DIPT). The observed mass for spot 15 was nearly half of the mass of the matching proteins of *C. sonorensis* (Q66U47_9DIPT, Q66U45_9DIPT, and Q66U43_9DIPT). The same protein also showed significant discrepancies for the pI data. Three different isoforms with pI values from 4.5 to 4.9 were found for one D7 related protein (Q66UB7_9DIPT), whereas the coagulation inhibitor (Q66U91_9DIPT) occurred in two different isoforms with pI data of 9.0 and 9.7. The calculated and observed molecular masses and pI data of proteins related to insects other than *C. sonorensis* were not always in agreement.

Skin Testing of Vasodilatory Activity. Intradermal testing of midge saliva was carried out with three human volunteers. At 30 min p.i., edema and erythema had developed at the injection sites. Edema reached a maximum at 1 h p.i., whereas the area of erythema extended until 4 h p.i. The diameter of skin reactions increased dose dependently as the concentration of saliva increased (Fig. 3). The diameters observed for the development of edema varied considerable between the test persons, whereas the development of erythema was more consistent. At 24 h p.i., edema and erythema had significantly decreased in all subjects and at all concentrations. Two of the three test subjects had developed strong pruritus at the site of injection. Pruritus was still observed 48 h p.i., whereas edema and erythema had disappeared. Neither erythema, edema, nor pruritus occurred at the injection site of the negative control (PBS).

Measurement of Human Plasma Clotting Activity. The recalcification time of PPP from three persons was tested in the absence and presence of midge saliva proteins. Untreated PPP showed clotting activity 5–6 min after the addition of calcium chloride. Increasing concentrations of saliva extended the clotting time in a dose-dependent manner (Fig. 4). Analysis of variance of the clotting activity of PPP in the absence and presence of midge saliva by using the software GraphPad Prism 4 (GraphPad Software Inc. 2003) showed *P*

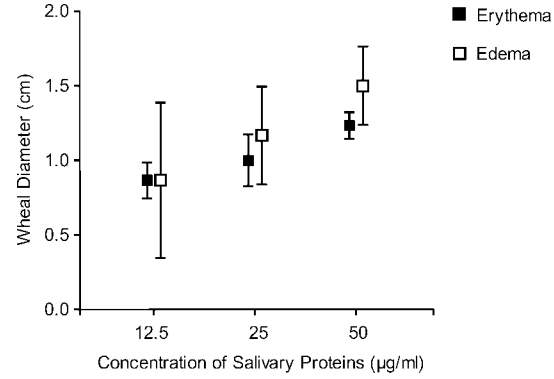

Fig. 3. Dose-dependent extent of cutaneous reaction by saliva of *C. nubeculosus*. Different concentrations of midge saliva were injected intradermally into the dorsal aspect of the arms of three human volunteers. Edema and erythema were measured at their maxima 1 h p.i., respectively, 4 h p.i. Results are expressed as the mean ± SEM.

values of 0.2283 (12.5 μg/ml), 0.0682 (25 μg/ml), and 0.0007 (50 μg/ml, highly significant).

Discussion

To date, the lack of efficient quantities of purified midge saliva has limited investigations of the salivary proteome of *Culicoides* spp. and the role of midge salivary proteins in pathogen transmission and hypersensitivity. In the current study, we developed a modified membrane feeding apparatus that allows the collection of significant amounts of midge saliva. In comparison with saliva collection from midges individually treated with parasympathomimetica (Boorman 1987, Fu 1995, Fu et al. 1999) and dissection of

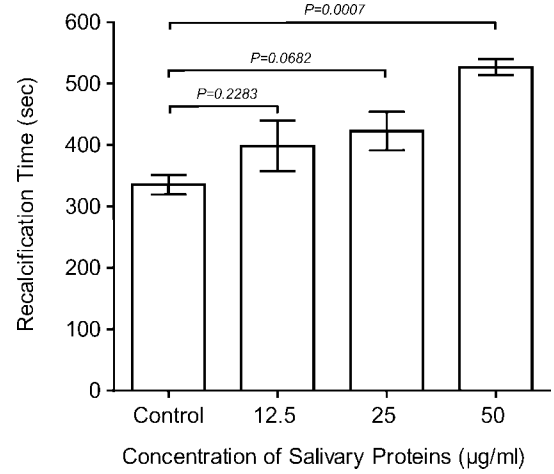

Fig. 4. Anticoagulant activity by saliva of *C. nubeculosus*. The recalcification time of human PPP was measured in absence (control) or presence of 12.5, 25, and 50 μg/ml midge saliva. Results are expressed as means ± SEM ($n = 3$). The comparison between means is indicated by horizontal bars; the italic numbers characterize the *P* values.

salivary glands (Perez de Leon and Tabachnick 1996, Perez de Leon et al. 1997, 1998), this method offers a more natural way of collecting saliva and prevents contamination with cellular proteins from salivary gland epithelial cells. Moreover, the time and level of training and skill required for obtaining the saliva with the modified membrane feeding apparatus is relatively low compared with the methods described previously.

The majority of the proteins analyzed from the saliva of *C. nubeculosus* showed high convergence with proteins of *C. sonorensis* recently identified from a salivary gland cDNA library. Maltases, together with amylases, and aldolases facilitate sugarmeal digestion. These enzymes have commonly been found in the salivary glands of several hematophagous insects such as mosquitoes and sand flies (Marinotti et al. 1996, Jacobson and Schlein 2001). In our investigations, we identified peptides from maltase but none from amylase or aldolase. This might be explained by previous findings of genomic approaches in *Anopheles* spp. and *C. sonorensis* that showed maltases and/or maltase-like 1α-glucosidases to be the most abundant members of the family of enzymes involved in sugarmeal digestion (Valenzuela et al. 2003, Campbell et al. 2005). The maltase-like α-glucosidase from *Aedes aegypti* (L.) saliva was identified as a major allergen in human hypersensitivity to mosquito bites (Peng and Simons 2004). Previous investigations in allergic horses revealed that skin reactivity is not only provoked by *Culicoides* bites but also by other hematophagous insects such as mosquitoes, which may indicate the presence of species-shared allergens (Geiben 2003). Because the peptide sequences from the maltase of C. *nubeculosus* not only show a high similarity to the maltase of *C. sonorensis* but also to the mosquito allergen, the midge maltase may be a candidate allergen in this insect known to be the primary cause of allergic dermatitis.

Proteins of the D7 family are present in the saliva or salivary glands of numerous female blood-sucking insects (James et al. 1991, Arcá et al. 1999, Valenzuela et al. 2002). The function of D7-related proteins is currently unknown. However, these proteins share some structural similarities to insect odorant-binding proteins and might function as small hydrophobic ligand carriers and/or in binding of host hemostatic factors (Valenzuela et al. 2002). Proteins of the D7 family are the most abundant proteins in the saliva of female mosquitoes (James et al. 1991). The predominance of the D7-related spots of *C. nubeculosus* suggests a similar abundance in midges. A D7 protein of *Ae. aegypti* is involved in mosquito allergies in humans (Peng and Simons 2004). Similarly, these proteins may function as allergens in horses.

Salivary proteins involved in defense protect arthropods against a variety of ingested and environmental pathogens. Antibacterial and antifungal compounds were identified in *Ae. aegypti*, *Triatoma infestans* Klug, and *Drosophila melanogaster* Meigen (Rossignol and Lueders 1986, Ferrandon et al. 1998, Amino et al. 2001). Additionally, the expression of defense molecules is up-regulated in different *Anopheles* spp. after the uptake of *Plasmodium* spp. (Dimopoulos et al. 1998, Shandilya et al. 1999). These molecules are assumed to induce parasite tolerance in the insects and to enhance the capability of parasite transmission. Potentially, defense molecules of *Culicoides* spp. could be involved in similar roles in pathogen transmission.

Screening of a salivary gland library from *C. sonorensis* revealed three platelet aggregation inhibitors and nine other coagulation inhibitors (Campbell et al. 2005). Surprisingly, only one Kunitz protease inhibitor-like protein was found in *C. nubeculosus* that is likely to occur in different isoforms. It is possible that artificial collection of saliva may result in the secretion of fewer platelet aggregation and coagulation inhibitor proteins than would be secreted when feeding on a host. However, prolonged clotting times were seen when human platelets were incubated with collected saliva, indicating the functional presence of these proteins (Fig. 4). Analysis of *C. sonorensis* salivary gland extracts revealed that antihemostatic proteins are efficient at the nanogram level (Perez de Leon and Tabachnick 1996). Because only the most abundant spots of *C. nubeculosus* were sequenced, proteins involved in coagulation inhibition may be among those not investigated. In addition, the proteins that were not identified or the proteins related to unclassified or novel midge proteins may have antihemostatic function. Salivary gland extracts of *C. sonorensis* were shown to inhibit blood coagulation by interference with extrinsic and intrinsic clotting pathways (Perez de Leon et al. 1998). Previously, an apyrase (platelet aggregation inhibitor) with an estimated molecular mass of 35 kDa and an inhibitor of factor Xa (inhibitor of the blood coagulation cascade) with an estimated molecular mass of 28 kDa were isolated by molecular sieving of salivary gland extracts (Perez de Leon and Tabachnick 1996, Perez de Leon et al. 1998). The coagulation inhibitor identified in this study had an observed molecular mass of 19 kDa. Thus, it is unlikely that the identified *C. nubeculosus* protein represents one of the previously described proteins, even when differences in molecular masses of species-specific variations are taken into consideration. Overall, proteins involved in the inhibition of blood coagulation are considered to be important for the exacerbation of infections with arthropod-borne pathogens and in the induction of allergic reactions (Titus and Ribeiro 1988, Perez de Leon et al. 1998, Valenzuela et al. 2002, Peng and Simons 2004). Therefore, the identified coagulation inhibitor may be a good candidate for further transmission and allergen investigations.

The peptide sequences related to proteins from insects other than *Culicoides* spp. might indicate the presence of novel midge salivary proteins. The potential function of a salivary carboxypeptidase is well described in ticks where it acts to destroy the pain mediator bradykinin, which is released subsequent to tissue damage (Ribeiro et al. 1985, Ribeiro and Mather 1998). Because *Culicoides* spp. feed on their hosts for only a few minutes and cause minor tissue damage in comparison with ticks, it may be unnecessary to eliminate the host's nociception. A more likely role is

protein digestion, identified for a carboxypeptidase recently isolated from the larval salivary glands of the wheat blossom midge, *Sitodiplosis mosellana* Gehin (Mittapalli et al. 2006). A similar function was suggested for the carboxypeptidases identified in the midgut of *C. sonorensis* (Campbell et al. 2005). Chemical sense-related lipophilic ligand binding proteins represent a subfamily of odorant binding proteins and have been identified in bees, beetles, and nonhematophagous flies (Hekmat-Scafe et al. 2002).

Alternatively, the peptides related to proteins from species other than insects most likely represent random hits. The observed identities to the plant species were relatively low, and no identities to insect proteins were found, even though sequences for similar proteins from different insect species were available on the databases.

Overall, the agreement of observed and theoretical molecular masses and pI data for the majority of proteins identified as putative midge salivary proteins illustrate the similarity between *C. nubeculosus* and *C. sonorensis*, which are both members of the subgenus *Monoculicoides.* Disagreement in observed and predicted molecular masses might be due to the dimerization or fragmentation of analyzed proteins or to species-specific differences. Differences in pI data could be attributed to posttranslational modifications or to multiple protein isoforms in addition to the explanations previously mentioned. The obvious differences of calculated and observed molecular masses and pI data of proteins related to insects other than *C. sonorensis* contribute to previous findings by Campbell et al. (2005) and are likely to be related to the independent evolvement of the midge's salivary factors.

Intradermal testing of the eluted salivary proteins showed strong evidence for the presence of vasodilatatory substances in *C. nubeculosus* saliva, even though no corresponding proteins were found by mass spectrometry analysis. Vasoactive proteins have previously been described for black flies, mosquitoes, and sand flies (Lerner and Shoemaker 1992, Ribeiro 1992, Perez de Leon et al. 1997, Cupp et al. 1998). Fractionating of salivary gland extracts from *C. sonorensis* by using a size exclusion column revealed maximal vasoactivity for proteins with molecular masses of ≈22.45 kDa (Perez de Leon et al. 1997). In addition to the vasodilatation, injection of the eluted salivary proteins produced edema and itching in the test subjects as described previously for midge bites in humans (Cohn 2003). Histamines, polyamines, and esterases capable of inducing these symptoms were isolated from the salivary glands of mosquitoes (Nakayama et al. 1985) and also might be present in the midge saliva. Moreover, intradermal application of *Culicoides* extracts is known to provoke allergic reactions in humans (Hoffman 1986). All volunteers were exposed to midge bites before the testing. Because the development of edema and pruritus varied considerably in the three subjects, the response may be due to sensitization to allergen(s) in the midge saliva rather than to direct impact of the injected saliva components.

In conclusion, we have developed a novel artificial collection method that allows the production of large amounts of salivary proteins of *Culicoides* spp. Saliva obtained with this method was used for the first analysis of salivary proteins of *C. nubeculosus.* In total, 12 proteins were related to salivary compounds of *C. sonorensis*, four of which have potential involvement in pathogen transmission and/or allergic reactions to midge bites. These proteins might be candidates for further investigations in both the vector competence of, and the hypersensitivity to, *Culicoides* spp. In addition, several peptides showed identities with proteins of species other than *C. sonorensis*, or they were not identified. Considering that *C. sonorensis*, compared with *C. nubeculosus*, is capable of transmitting many arboviral livestock pathogens, these differences may represent significant grounds for investigation with regard to either vector competence or in defining components that modulate host immune responses. Functional properties of the saliva were preserved as illustrated by skin reactivity and hematostasis. Therefore, salivary proteins collected in this way may be used for investigations of their effects on arbovirus infectivity and transmission as well as for studies on equine allergy, elucidating the role of the individual components involved in the induction of summer eczema.

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